

Advanced Glycation End Products (AGEs) Antibody Protects Against AGEs-Induced Apoptosis and NF- κ B p65 Subunit Overexpression in Rat Glomerular Culture

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ABSTRACT

Advanced glycation end products (AGEs) have been thought to be a major cause of diabetic nephropathy (DN). The mechanisms underlying the involvement of AGEs antibody in diabetic nephropathy are not fully understood. The present study was designed to investigate the protective effect of AGEs antibody on AGEs-induced glomerular damage. Isolated glomeruli were pre-incubated either with 10 μ g/mL polyclonal anti-AGEs antibody (AGE-pAb) or monoclonal anti-N³-carboxymethyl-lysine antibody (CmL-mAb) as a model of AGEs antibody to block interaction of AGEs with receptor for AGEs (RAGE) and incubated afterwards either with 100 μ g/mL bovine serum albumin (BSA) or AGE-modified bovine serum albumin (AGE-BSA) for 48 h. Annexin V/nephrin double-staining was performed to determine apoptosis. Using immunofluorescence, we found that administration of AGE-BSA not only significantly increased glomerular cells apoptosis and nuclear factor kappa B (NF- κ B) p65 expression, but also reduced expression of nephrin, an important structural and signal molecule of podocytes slit diaphragm. Blocking the interaction of AGE-RAGE with AGEs antibody significantly protected glomerular cells from AGEs-induced apoptosis and NF- κ B p65 overexpression. We found that AGE-pAb conferred superior protective effect compared with CmL-mAb for the same reduction in apoptosis and NF- κ B p65 expression. In sharp contrast, CmL-mAb led to preserve expression of podocytes nephrin better than AGE-pAb. These results demonstrate that the antibody against AGEs may be beneficial for preventing the glomerular damage in DN.

Keywords: Advanced glycation end products; antibody, apoptosis, diabetic nephropathy, N³-(carboxymethyl)lysine

INTRODUCTION

Diabetes mellitus (DM) is a significant health problem with a worldwide mortality around 382 million people in 2013, and this number is expected to rise to 592 million by 2035 [1]. AGEs are a causative factor in diabetic vascular complication such as diabetic nephropathy (DN) which further lead to end-stage renal disease [2,3]. Accumulation of AGEs is present in all renal compartments in diabetic patient with three mechanisms of toxicity include interaction AGEs with receptor for AGE (RAGE), *in situ* glycation and tissue deposition [4]. Inhibition of AGEs is widely regarded as an implicit goal in clinical medicine for the treatment of DN [5]. So far, most studies have been focused on the potential of therapies that not only target

various pathways upregulated by AGEs, but also target AGEs itself by preventing AGE formation, breaking AGE-protein cross-linking, or neutralizing AGE [6–11]. In addition to the development of anti-AGE activity from natural product or synthetic compound, immunization of AGEs in diabetic mice has recently attracted attention [12].

AGEs have antigenic properties that may exert an autoimmune response. Autoantibodies against AGEs are detected in serum of patients with DM and non-DM which may play a role in the macrophage uptake of AGEs-modified protein via AGEs-immune complexes (AGE-IC) formation [13,14]. Disruption of the balance between AGEs formation and AGEs elimination will lead to accumulation of AGEs [14,15]. How-

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ever, our knowledge of the molecular mechanism governing role of AGE-Ab and AGE-IC in diabetic complication is still very limited. Thus, in this research, we aimed to demonstrate the role of AGE-Ab in prevent AGEs-induced glomerular damage.

MATERIALS AND METHODS

Ethics

This research was approved by the Health Research Ethics Commission from Faculty of Medicine, Brawijaya University, Malang, Indonesia, with registration number 356B/EC/KEPK-S2/06/2015.

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. AGE-mAb was purchased from Circulex (Nagano, Japan). AGE-pAb was purchased from Abcam (Cambridge, England). BSA (fraction V) was purchased from Roche Diagnostic GmbH (Mannheim, Germany). AGE-BSA was purchased from BioVision (California, USA). For immunofluorescence assay, the following primary antibodies were used: rabbit polyclonal anti-NF- κ B p65 (Bioss, Woburn, USA), goat monoclonal anti-nephrin (Santa Cruz Biotechnology, California, USA), rabbit polyclonal anti-nephrin (Bioss, Woburn, USA) and Annexin V conjugated fluorescein-isothiocyanate (FITC) (BioLegend, Fell, Germany). Secondary antibodies were used: goat anti-rabbit IgG-FITC, rabbit anti-goat IgG-rhodamine and goat anti-rabbit IgG-rhodamine (Santa Cruz Biotechnology, California, USA). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA).

Rat glomerular isolation and culture

Isolation and culture of glomeruli were done as described previously with some modifications [16–19]. Briefly, male Wistar rats at age 7–9 weeks were sacrificed by cervical dislocation and perfused with ice-cold Hank's balanced salt solution (HBSS) through the heart. The kidneys were perfused with ice-cold HBSS and dissected into small pieces (1–2 mm³ cubes) with a surgical blade in ice-cold HBSS. Next, the tissues were digested in collagenase solution containing 1 mg/mL collagenase A in HBSS at 37°C for 1 hour with gentle agitation. The collagenase-digested tissues were gently pressed through a 100 μ m cell strainer (BD Bioscience, Bedford, USA), followed by flushing with 5 mL of ice-cold sterile HBSS. The cell suspension was then centrifuged at 200 \times g for 5 minutes. The supernatant was discarded and the cell pellet was resus-

pended in 2 mL of ice-cold HBSS. Glomeruli were isolated manually with micro-hematocrit tube and transferred at least two times into another dish to remove any remaining debris which included when isolation process. All procedure were performed on ice, except for the collagenase digestion. Isolated glomeruli were cultured on type I collagen-coated glass-bottom culture dishes (MatTek Corporation, Ashland, MA) in RPMI 1640 medium containing 10% FBS supplemented with 10% penicillin-streptomycin and 1% insulin-transferin-selenium A liquid media supplement. Glomeruli were cultured at 37°C in a moist 95% air/5% CO₂ atmosphere.

Incubation of glomeruli with AGEs and blocking with AGEs antibody

Treatment was performed after 3 days of culture of isolated glomeruli. To block the binding of AGEs to RAGE, 10 μ g/mL AGE-pAb or AGE-mAb pre-incubated for 1 hour before 100 μ g/mL BSA or AGE-BSA were added for 48 hours. All experiments were repeated two times in each indicated condition.

Double labelling immunofluorescence staining

The indirect immunofluorescence technique was applied to fixated glomeruli. Double labelling of cultured glomeruli was performed to evaluate the effect of AGE-Ab on AGEs-induced apoptosis and NF- κ B p65 expression. In brief, after being incubated with AGE-BSA in the absence or pre-treatment of AGE-Ab with a 1 hour interval, glomeruli on glass bottom dish were fixed with 4% paraformaldehyde for 15 minutes, washed with PBS three times, permeabilized with 2% Triton X-100 for 5 minutes, blocked with blocking solution (2% BSA and 2% FBS in PBS) for 30 minutes, and incubated with primary antibody for 1 hour. For double-label immunostaining of NF- κ B p65 expression, anti-NF- κ B p65 and anti-nephrin primary antibody were premixed as follows and applied simultaneously. After washing with PBS three times, glomeruli were incubated with fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary antibody for 1 hour, then washed again with PBS three times. Sequential double staining was performed to evaluate apoptosis using FITC-conjugated annexin V for 1 hour, blocked with blocking solution for 30 minutes, incubated with anti-nephrin primary antibody for 1 hour, and then incubated with rhodamine-conjugated secondary antibody for 1 hour. The negative control was performed using 2% BSA in PBS instead of the primary antibody.

Quantitative imaging

Fluorescence images were acquired using confocal laser scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan) and recorded on a computer using the Olympus Fluoview ver 1.7a viewer. The expression of protein was determined as described previously [19]. Digital pictures of the green and red channels were quantitated using the ImageJ 1.50 software (NIH, MD, USA). Three fields were selected for analysis of each stain. The images were converted to 8-bit grayscale. Then, an outline was drawn around each glomeruli and selected as the region of interest (ROI) to measure area (A) and integrated density (ID) of fluorescence. Next, the mean gray value of background readings (MGV) was measured by selecting five distinct areas in the background with no fluorescence. The corrected optical density (COD) = $ID - (A \times MGV)$, was calculated. Data were plotted using Prism 6.0 software (GraphPad, San Diego, CA, USA).

Statistical analysis

All data are presented as mean \pm SD of two independent experiments. The statistical analysis was performed using the IBM SPSS Statistics 20 software for Windows (IBM Corp., Armonk, NY, USA). Differences between groups were determined by Kruskal-Wallis tests, followed by Mann-Whitney post-tests to calculate statistical significance. Correlation analyses between variables were evaluated using the non-parametric Spearman rank coefficient. Group differences at the level of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The previous study demonstrated that AGEs accumulation resulted in glomerular damage by activating RAGE in podocyte, endothelial cells, mesangial cells, and tubular cells [4]. In this study, we provided in vitro evidences that blocking AGE-RAGE interaction with AGEs antibody protects glomerular cells from apoptosis and NF- κ B overexpression induced by AGEs. To the best of our knowledge, this is the first study demonstrating that AGEs antibody protects glomerular damage induced by AGEs. Previously study demonstrated that AGE-RAGE interaction can also be inhibited by pre-incubation of RAGE-Ab. This antibody protects functional and morphological damages of human podocytes induced by peritoneal dialysis fluid- and glucose degradation products [20].

AGEs antibody down-regulated AGEs-induced glomerular NF- κ B p65 overexpression

To examine the effect of AGEs on NF- κ B p65 expression in glomerular cells, we pre-incubated glomeruli with AGE-pAb or AGE-mAb before AGE-BSA exposure. As shown in Figure 1A-B, AGEs significantly increased NF- κ B p65 expression in cultured glomeruli. However, AGEs-induced over-expression of NF- κ B p65 was significantly inhibited by preincubation of AGE-Ab. AGE-pAb significantly decreased NF- κ B p65 expression better than AGE-mAb. Since nephrin expression has been associated with podocyte survival, we studied the effect of AGE-Ab on nephrin expression. The presence of AGE-BSA significantly reduced nephrin expression, thus increased NF- κ B/nephrin expression ratio (Figure 1C). Both AGE-pAb and Cml-mAb significantly reduced NF- κ B/nephrin expression ratio compared with AGE-BSA-treated group. These findings indicated that AGE-Ab down-regulated AGEs-induced NF- κ B p65 over-expression.

AGEs are believed to play a major role in the development of DN. Interaction of AGE-RAGE activates a series of intracellular signaling pathway, including NF- κ B transcription factor which initiates and stimulates the production of pro-inflammatory molecules that contribute to the pathogenesis of DN [3]. Our study showed that AGE-BSA increased glomerular NF- κ B p65 expression. This finding supported by Bierhaus et al. [21] that binding AGEs to RAGE results in sustained activation of NF- κ B as the result of increased levels of de novo synthesized NF- κ B p65. Sustained activation NF- κ B is mediated by initial degradation of I κ B protein followed by new synthesis of NF- κ B p65 mRNA and protein in the presence of newly synthesized I κ B α and I κ B β [21]. Research conducted by Peng et al. [22] showed that AGEs signal via RAGE generates a signal-specific post-translational modification, or a "barcode" to NF- κ B that mediates a specific gene expression pattern. AGE-RAGE signaling results in NF- κ B activation via phosphorylation of NF- κ B p65 subunit at T254, S311, S536 residues [22]. AGEs also induces NF- κ B activation mediated by suppression of sirtuin 1 (SIRT1) expression in podocytes, which leads acetylation of NF- κ B p65 subunit [23].

Unlike many other receptors, the activation of RAGE by AGEs positively upregulates RAGE expression, resulting in sustained RAGE signaling through a NF- κ B-dependent mechanism [24,25]. Moreover, activation of NF- κ B also upregulates AGEs formation via

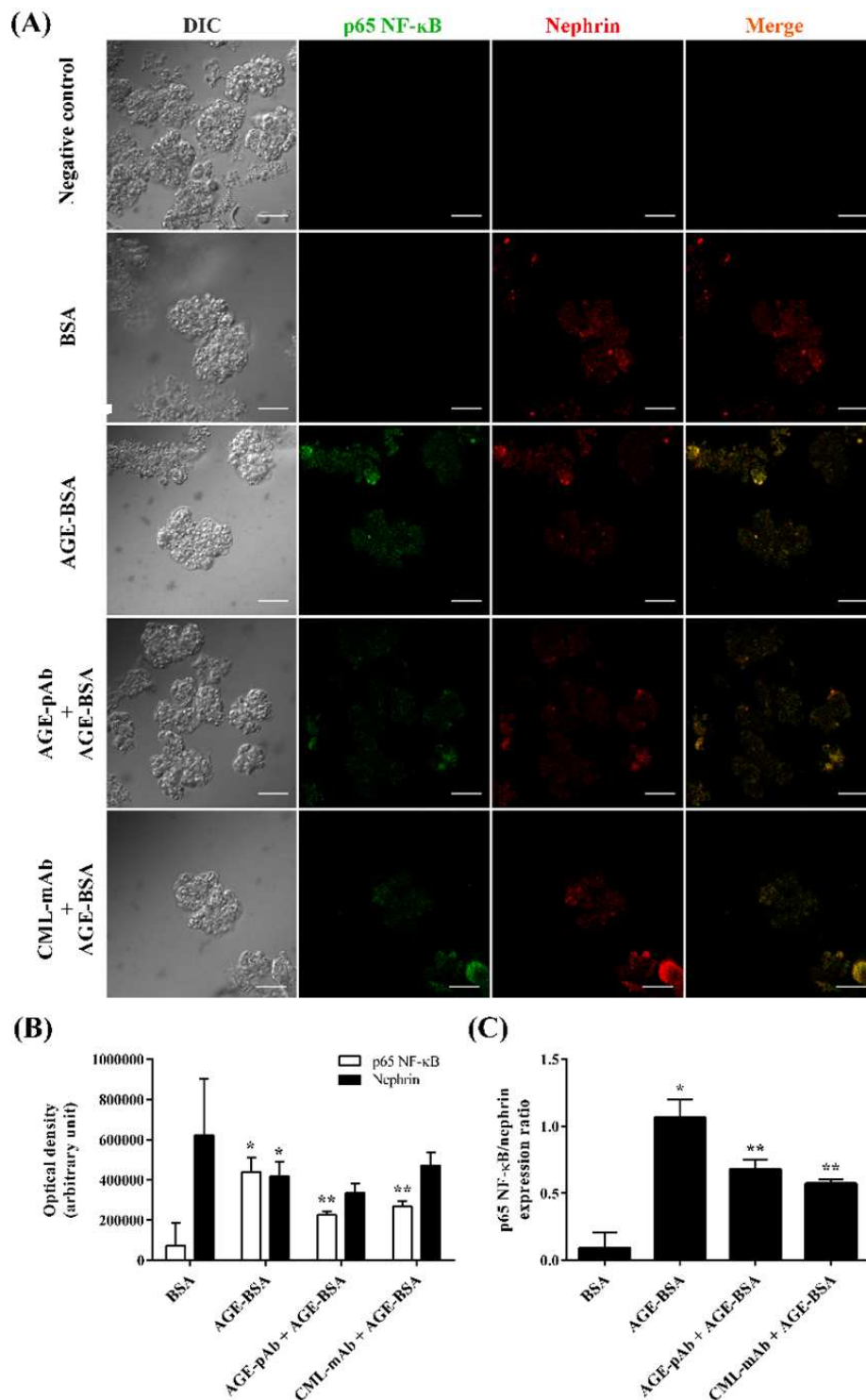


Figure 1. AGEs antibody downregulated glomerular NF- κ B p65 expression induced by AGEs. A. Representative confocal microscopic images of NF- κ B p65 (green) and nephrin (red) fluorescence in the glomeruli. BSA was chosen as normal control while AGE-BSA as positive control. The primary antibody was replaced by 2% BSA in PBS for negative control. Magnification 400 \times . Scale bars = 50 μ m. B. Bar graphs show the average optical density of NF- κ B p65 and nephrin per glomerular. C. Expression ratio of NF- κ B p65/nephrin. Data were expressed as mean \pm SD. AGE = advanced glycation end product; AGE-pAb = polyclonal anti-AGEs antibody; BSA = bovine serum albumin; CmL-mAb = monoclonal anti-N ϵ -carboxymethyl-lysine antibody; NF- κ B = nuclear factor-kappa B. * p < 0.05, vs BSA; ** p < 0.05, vs AGE-BSA.

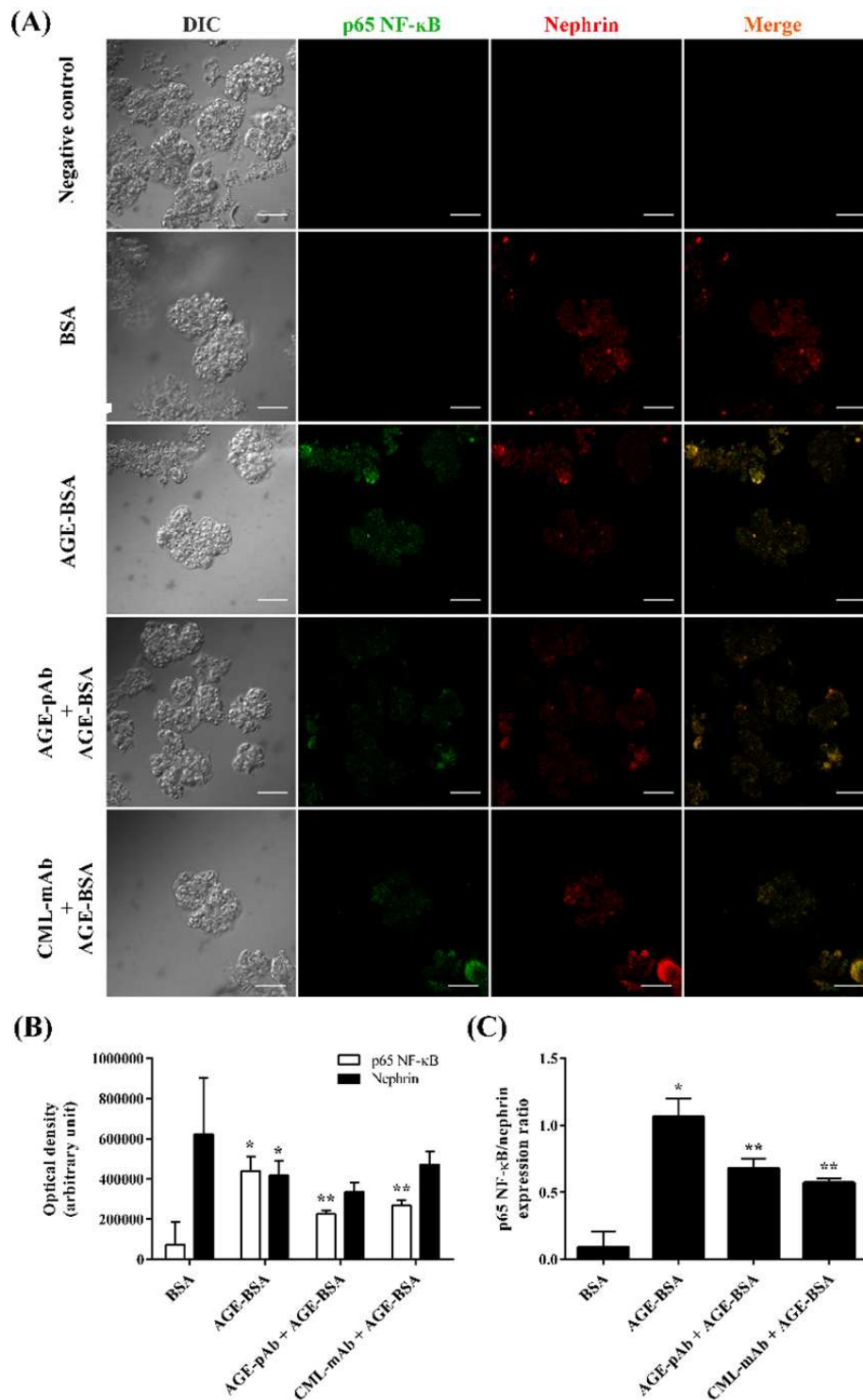


Figure 2. AGEs antibody ameliorated glomerular cells apoptosis induced by AGEs. A. Images show glomeruli in either normal condition or treated with AGE-BSA for 48 hours in the absence or presence of AGE-Ab. Apoptotic cells were stained with FITC-conjugated Annexin V (shown in green) due to phosphatidylserine externalization and nephrin (shown in red). Magnification 400 \times . Scale bars = 50 μ m. B. Optical density of annexin V and nephrin was calculated using ImageJ. C. Expression ratio of annexin V/nephrin. Data were expressed as mean \pm SD. AGE = advanced glycation end product; AGE-pAb = polyclonal anti-AGEs antibody; BSA = bovine serum albumin; Cml-mAb = monoclonal anti-N ϵ -carboxymethyl-lysine antibody. * $p < 0.05$, vs BSA; ** $p < 0.05$, vs AGE-BSA.

suppressing the expression of glyoxalase which inactivates the AGE precursor methylglyoxal [25]. RAGE activation in mesangial cells increases angiotensin-II (Ang-II) production then activates transforming growth factor- β -Smad signaling that induces mesangial cell hypertrophy [26]. In addition, Ang-II induces podocyte injury and nephrin inactivation (dephosphorylation) through caveolin-1-dependent mechanism and C-terminal-Src kinase- C-terminal-binding protein-Fyn axis [27,28]. Reduction of nephrin surface expression is also induced by activation of protein kinase C-mediated nephrin endocytosis [29]. Nephrin deficiency activates NF- κ B and disrupts clustering of membrane raft micro-domains to prevent nephrin-mediated signaling, which affects the podocytes or other glomerular cells that interact with podocytes [30,31].

AGEs antibody ameliorated AGEs-induced apoptosis of glomerular cells

Figure 2A-B illustrate the effect of AGEs and AGE-Ab on apoptosis. AGE-BSA treatment resulted in a significant increase of glomerular cells apoptosis and decrease of podocyte nephrin which significantly inhibited by pre-incubation of AGE-Ab. Previously study showed that AGE-BSA induces apoptosis by enhancing FOXO4 binding to a forkhead binding element in the promoter of *Bcl2l1* and increasing the acetylation of FOXO4 at lysine residues mediated by downregulation of sirtuin 1 (SIRT1) in cultured podocytes and in glomeruli of diabetic patients [32]. Likewise, AGE-Ab significantly reduced apoptosis/ nephrin expression ratio compared with AGE-BSA-treated group (Figure 2C). Interestingly, we found that nephrin expression significantly preserved in the group of glomeruli under AGE-mAb-treated group better than AGE-pAb-treated group. These results suggested that AGE-Ab had a protective activity on AGEs-induced glomerular cells apoptosis and nephrin depletion.

The correlation between NF- κ B p65 expression and apoptosis

A positive correlation was observed between NF- κ B p65 expression and apoptosis ($r = 0.818$) as well as between NF- κ B/nephrin and apoptosis/nephrin expression ratio ($r = 0.828$). Taken together, these data provided indirect evidence that AGE-Ab-mediated down-regulation of NF- κ B p65 expression may contribute to inhibition of glomerular cells apoptosis. The results of the present study support the protective role of AGE-Ab against glomerular cells apoptosis, NF- κ B p65 overexpression and nephrin depletion induced by AGEs. In

this study, we found that polyclonal anti-AGEs antibody protects apoptosis and NF- κ B p65 overexpression better than monoclonal anti-CmL antibody. We speculate that AGE-IC formation contributes to these effects. According to Mera et al. [13], we hypothesize that polyclonal AGEs antibody recognizes multiple epitopes on AGE-BSA, thus increases the formation of AGE-IC which induces macrophages-mediated AGE-BSA phagocytosis [13]. However, activated macrophages can downregulate nephrin expression [33]. In this study, monoclonal CmL-specific antibody preserves nephrin expression. CmL is one of the major immunogen of AGEs that accumulates in all renal compartment of diabetic patient. CmL increases expression of ZEB2 by NF- κ B activation and results in epithelial-mesenchymal transformation of podocyte which is believed to play a vital role in podocyte depletion and the pathogenesis of albuminuria during DN [34]. Our results showing only minor reduction of nephrin expression on pre-incubation of polyclonal anti-AGEs antibody. The monoclonal anti-CmL antibody obviously exerts protective effect to podocyte with an aim to preserve nephrin expression. Previously study reported that both polyclonal and monoclonal anti-CmL antibody have a significant reactivity to CmL-proteins. Moreover, this monoclonal antibody significantly reacted with AGE-BSA as well as BSA modified by several aldehydes such as glyoxal [35].

CONCLUSION

In conclusion, the present data confirm that pre-incubation of AGEs antibody inhibits apoptosis and NF- κ B p65 overexpression in glomeruli exposed to AGEs. Our study demonstrates for the first time that the antibody against AGEs may prevent AGEs-induced glomerular damage.

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